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TITLE: **ULTRAFast AND NOT-SO-FAST DYNAMICS OF CYTOCHROME
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RELEVANCE TO THE REDOX-LINKED PROTON PUMP**

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ULTRAFAST AND NOT-SO FAST DYNAMICS OF CYTOCHROME OXIDASE: THE LIGAND SHUTTLE AND ITS POSSIBLE FUNCTIONAL SIGNIFICANCE

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ABSTRACT

Time-resolved electronic absorption, infrared, resonance Raman, and magnetic circular dichroism spectroscopies are applied to characterization of the intermediate which is formed within 20 ps after photodissociation of CO from cytochrome a₃ of reduced cytochrome oxidase. This intermediate decays with the same half-life (ca. 1 μs) as the post-photodissociation Cu_B⁺-CO species previously observed by time-resolved infrared (1). The transient UV-Vis spectra, kinetics, infrared, and Raman evidence suggest that an endogenous ligand is transferred from Cu_B to Fe_{a3} when CO binds to Cu_B, forming a cytochrome a₃ species with axial ligation which differs from the reduced unliganded enzyme. The time-resolved magnetic circular dichroism results suggest that this transient is high spin and therefore five coordinate. Thus we infer that the ligand from Cu_B binds on the distal side of cytochrome a₃ and displaces the proximal histidine imidazole. This remarkable mechanistic feature is an additional aspect of the previously proposed (1) "ligand shuttle" activity of the Cu_B/Fe_{a3} pair. We suggest that the ligand shuttle may play a functional role in redox-linked proton translocation by the enzyme. More detail on this work is presented elsewhere (1-5).

1. INTRODUCTION

In recent studies of the events following photodissociation of CO from cytochrome oxidase (CcO) (1-3), we reported the first conclusive evidence that, at room temperature, CO binds to Cu_B⁺ both entering and leaving its thermodynamically stable binding site on Fe_{a3}²⁺. This mechanism is depicted in Scheme 1. In this scheme K_1 and k_2 are 130 M⁻¹ and 700 s⁻¹, respectively (2). Using time-resolved infrared (TRIR) spectroscopy, we have determined k_1 to be 4.7×10^5 s⁻¹ (1). These results reveal that Cu_B⁺ can function as a "ligand shuttle" in transporting exogenous ligands (perhaps including O₂) to and from the heme of cytochrome a₃. Here we present results which suggest that, during the lifetime of the Cu_B⁺-CO complex, the cytochrome a₃ heme differs from the 5-coordinate, imidazole-liganded, high-spin structure that it assumes in the reduced unliganded enzyme. The probes that we have applied (1-3) include transient UV-Vis, TRIR, time-resolved resonance Raman (TR³), and time-resolved magnetic circular dichroism (TRMCD). The results suggest a remarkable feature of the ligand shuttle reactivity of Cu_B/Fe_{a3}. It appears that an endogenous ligand, which we denote L, in binding to cytochrome a₃ on the distal side, displaces the proximal histidine resulting in a transient 5-coordinate species, Fe_{a3}-L. Furthermore, it appears that L is not imidazole. Given the similar properties of O₂ and CO as ligands, it seems probable that similar ligand shuttle phenomena may accompany the coordination of O₂ before its reduction and may play a significant role in the functional dynamics of the enzyme.

Scheme 1

2. RESULTS AND DISCUSSION

Transient Absorbance. Using UV-Vis measurements between 100 fs and 100 ns, we have studied the photodissociation/recombination reactions of $\text{Cytochrome } a_3\text{-CO}$ (3,4). Subpicosecond spectroscopy (4) reveals the ultrafast events at both cytochrome a_3 and $a_3\text{-CO}$. Picosecond transient difference spectra are shown in Figure 1 for both the unliganded reduced enzyme and the CO complex. In the latter (Figure 1a), the timescale of the initial cytochrome a_3 photoevent (CO dissociation) is probably close to the Fe-C vibrational period, ca. 64 fs. Figure 1b exhibits only cytochrome a_3 transients. These include prompt (< 100 fs) formation of an excited state which decays in ca. 4 ps to an intermediate that we believe to be a 5-coordinate ground state species. The original 6-coordinate ground state is then reformed with a half-life of 20 ps. Figure 1b shows a good isobestic point at 610 nm which Figure 1a lacks. This appears to be due to an increase in the cytochrome a_3 α band intensity at this wavelength subsequent to the dissociation of CO. A possible reason for this change is suggested below.

The dependence of the millisecond-timescale reaction for rebinding CO to the heme (rate constant k_{obs}) upon $[\text{CO}]$ yields the rate constant k_2 for transfer of CO from Cu_B^+ to Fe_{a3}^{2+} , 700 s^{-1} and the pre-equilibrium constant K_1 , 130 M^{-1} (3). On shorter timescales the α -band shows two additional transients. The first is an increase in absorbance with a half-life of ca. 10 ps, noted above (4). By comparison, the risetime of the $\text{Cu}_B\text{-CO}$ absorbance measured by TRIR is 1 ps or less (5). The second is a decrease in ΔA with the same rate constant as the $5 \times 10^5 \text{ s}^{-1}$ observed by TRIR for loss of CO by $\text{Cu}_B^+ \cdot \text{CO}$ (1). We infer from these results that a ligand L is transferred with a 10 ps half-life from Cu_B^+ to cytochrome a_3 , triggered by the 1 ps binding of photodissociated CO to Cu_B^+ . Conversely, we suggest that L returns from cytochrome a_3 to Cu_B in ca. 1 μs , triggered by loss of CO from $\text{Cu}_B^+ \cdot \text{CO}$ into solution. We have also observed that the recombination of CO with cytochrome a_3 is accelerated by increasing the probe light intensity of the transient spectrophotometer. This is easily understood in the mechanism suggested above. If breaking of the Fe-L bond is the rate determining step for the return of CO to the heme, and if the Fe-L bond is photolabile, light will accelerate both k_2 and k_{obs} .

Time-Resolved Resonance Raman: The TR³ spectra shown in Figure 2 were obtained at pulse-probe time delay of 200 ns; both high probe laser pulse energy (1 mJ) and low energy (ca. 10 μJ) were employed. In the high-frequency region some diminution in intensity of the high-spin indicator band (1572 cm^{-1}) occurs at low probe pulse energy. At the same time, the oxidation state marker peak appears

Figure 1. Picosecond transient difference electronic spectra of fully reduced beef heart cytochrome oxidase: a. CO derivative; b. unliganded enzyme. Photodissociation wavelength, 595 nm; pulse duration, 150 fs. The numerals are nominal pump-probe delay times in picoseconds, but the white light probe pulse is subject to group velocity dispersion such that the red end of each of the two spectral ranges shown (400-540 nm and 540-680 nm) represents times ca. 4 ps earlier than the blue end.

Figure 2. TR³ spectra of CcO-CO in the high-frequency and low-frequency regions taken 200 ns after photodissociation of CO. In each frequency region the upper traces are taken using ca. 1 mJ/pulse of probe laser light at 441 nm and the lower traces are taken using 10 μ J/pulse.

unchanged in frequency and lineshape at 1356 cm^{-1} . This demonstrates that the low-power spectrum is not due to the CO complex, which would show an oxidation state marker peak at ca. 1372 cm^{-1} . In the low-frequency region the high-power spectrum clearly shows the Fe-N(Im) stretch of 5-coordinate high-spin cytochrome a₃ at 223 cm^{-1} , as in an earlier TR³ study (6). However, this peak is missing in the low-power trace. The low-power TR³ spectrum could be due to a 6-coordinate low-spin species with both L and imidazole as axial ligands, wherein the Fe-N stretch is missing as it usually is in low-spin heme spectra. Alternatively, it may be due to a 5-coordinate high-spin cytochrome a₃-L complex wherein L is not imidazole and has properties which induce cleavage of the Fe_{a3}-N(proximal histidine) bond. In this interpretation the disappearance of the 223 cm^{-1} peak occurs simply because the axial ligand L is not imidazole.

Time-Resolved Magnetic Circular Dichroism: The MCD spectrum of reduced unliganded CcO has a large contribution from high-spin cytochrome a₃. This is characterized by an intense positive Soret MCD feature at 455 nm. In the TRMCD a signal which is similar to that of unliganded CcO develops within the excitation laser pulsewidth (≤ 10 ns), and remains the same until replaced by that of CcO-CO on the millisecond timescale. The TRMCD results are strong evidence that the transient observed in the UV-Vis and TR³ experiments is high spin.

Nature of the 10 ps - 1 ms Transient and Possible Identity of Ligand L: The evidence presented above favors a structure for the post-photodissociation transient wherein CO is bound to Cu_B, L is released and binds to cytochrome a₃ on the distal side, and the bond between the heme iron and the proximal histidine is broken. The UV-Vis kinetics (3) set the timescales for the binding of L to the heme (≤ 20 ps) and its return to Cu_B (~ 1 μ s) as CO binds to Cu_B (<1 ps) and dissociates into solution (~ 1 μ s) (1,5). The TRMCD suggests that the transient is high spin, therefore 5-coordinate. While we cannot definitively reject a 6-coordinate high-spin structure, this seems an unlikely alternative based upon the spectroscopic and kinetics evidence and existing model data.

Conclusions: Figure 3 presents a minimal mechanism needed to account for the kinetics and the spectroscopic facts (ignoring for the moment the protonation/deprotonation reactions, which are included for heuristic purposes detailed below). The ligand L is exchanged between Cu_B⁺ and Fe_{a3}²⁺ as CO binds to and dissociates from Cu_B. The binding of L to Fe_{a3} causes the release of the proximal histidine. The five-coordinate Fe_{a3}²⁺-L complex, which is produced by routes which differ in detail depending upon whether CO dissociates thermally from the heme, or the heme and the CO carry the excess energy of

photoexcitation, is suggested to be responsible for the short timescale α -band transients, the TRMCD spectra, and the low-power TR³ spectrum.

We expect that the phenomena observed in the coordination dynamics of CO are representative of the behavior of other similar π -acceptor ligands, including NO, isocyanides, and most importantly O₂. If this is the case the "ligand shuttle" activities of Cu^{B+} (i) in acting as an intermediate binding site for ligands entering and leaving the site of O₂ reduction, (ii) in synchronously transferring L between Cu^B and cytochrome a₃, and (iii) in engendering the release and rebinding of the proximal histidine imidazole, undoubtedly have functional significance. One such function of the ligand shuttle is suggested (schematically and speculatively, presuming L to be a group with a labile proton) in Figure 3. Directed protonation/deprotonation reactions of L and the proximal histidine, accompanying the transfer of L from one metal to another on one side of the heme plane and the release and rebinding of the proximal histidine on the other, may act as microscopic "gates" for active proton translocation. The proton gating mechanism based upon the ligand shuttle embodies the novel feature of being linked to the coordination chemistry of the Cu^B/Fe_{a3} pair, rather than exclusively to the redox reactivity.

We emphasize that the thermodynamic driving force for proton translocation must arise from the energy of the electron transfer reactions associated with the reduction of O₂, as is commonly recognized (7). However, the ligand shuttle as a proton gate may be coupled to electron transfer in a very straightforward way, namely by changes in heme and copper redox potentials and electron transfer rates as a consequence of changing the identity or protonation state of the ligands. In this regard we note the recent result of Brzezinski and Malmström (8), who reported the electron transfer kinetics of mixed-valence CcO following photodissociation of CO from Fe_{a3}²⁺. The authors established the rate constant for electron transfer from (Fe_{a3}²⁺, Cu^{B+}) to Cu^{A2+} in the "E1 state" to be 600 s⁻¹ ($\Delta H^\ddagger = 7.7$ kcal/mol, $\Delta S^\ddagger = -18$ eu). By comparison we have measured the rate of dissociation of the Fe_{a3}²⁺-L bond (k_2 in Scheme 1 or the conversion of intermediate 2 to 3 in Figure 1) to be 700 s⁻¹ ($\Delta H^\ddagger = 10$ kcal/mol, $\Delta S^\ddagger = -12$ eu) (3). The similarity of these kinetics parameters suggests that the rate determining step for the electron transfer reaction may be the cleavage of the Fe_{a3}²⁺-L bond, as suggested in Figure 4. This suggests that a major microscopic difference between the E1 and E2 proton translocating states postulated by Brzezinski and Malmström (7b, 8) may be whether L is bound to Fe_{a3} or Cu^B.

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Figure 3. Pictorial representation of the mechanism of CO binding to and dissociation from cytochrome oxidase. Only Cu(I) and Fe(II) are shown. The species 1, 2, 3, 4, and 5 represent the thermal mechanism. All of the reactions are first order, except for the bimolecular reaction with CO in solution; the half-lives are either known or reliably estimated from experiment, or else they are omitted. The protonation/deprotonation reactions shown are hypothetical, not inferred from the evidence presented here (see text).

Figure 4. Suggested mechanism for the electron transfer reaction from Fea3 to CuA following photodissociation of the mixed-valence CO complex of cytochrome oxidase. See "conclusions" section of the text and Reference 7. The species 1, 2, 3, 4, and 5 are the mixed-valence analogues of the corresponding species in Figure 1.